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Analyzing Phosphorylation-Dependent Regulation of Subcellular Localization and Transcriptional Activity of Transcriptional Coactivator NT-PGC-1 α

Ji Suk Chang and Thomas W. Gettys

Abstract

Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α) is a nuclear transcriptional coactivator that regulates the genes involved in energy metabolism. Recent evidence has been provided that alternative splicing of PPARGC1A gene produces a functional but predominantly cytosolic isoform of PGC-1 α (NT-PGC-1 α). We have demonstrated that transcriptional coactivation capacity of NT-PGC-1 α is directly correlated with its nuclear localization in a PKA phosphorylation-dependent manner. In this chapter, we describe quantitative imaging analysis methods that are developed to measure the relative fluorescence intensity of the protein of interest in the nucleus and cytoplasm in a single cell and the frequency distribution of nuclear/cytoplasmic intensity ratios in the population of cells, respectively. This chapter also describes transient cotransfection and dual-luciferase reporter gene assay that examine the ability of coactivators to activate the transcriptional activity of transcription factors.

Keywords

Coactivator; PGC-1 α ; NT-PGC-1 α ; Nuclear receptor; PPAR γ ; Transcription

1. Introduction

Transcriptional activity of nuclear receptors and transcription factors is dynamically regulated in response to physiological stimuli by transcriptional coregulators such as coactivators and repressors. PGC-1 α is one such protein that activates multiple nuclear

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¹Filtration of 5 $\times$ Blocking solution (5% NGS and 5% BSA) through a 0.22 μ m filter significantly reduces tiny spot-like fluorescent backgrounds.

²Transfection efficiency of CHO-K1 using FuGENE 6 is very low when >60% confluent. It is recommended to seed CHO-K1 cells at ~40% confluency to get ~50% transfection efficiency. Also, seeding the cells at low confluency allows single cells to nicely spread out without contacting each other 24 h after transfection.

³The biological activity of undiluted FuGENE6 Reagent is significantly reduced by the contact with plastic surface. Thus it is critically important to avoid the contact of FuGENE 6 Reagent with plastic walls of the tube containing Opti-MEM I medium during the dilution step. Always add FuGENE 6 Reagent by pipetting directly into the Opti-MEM I medium.

⁴In the presence of serum, CHO-K1 cells tend to be round up on a poly-L-lysine-coated or non-coated coverslip. Serum starvation allows a well-spread out morphology of CHO-K1 cells on a coverslip.

⁵Repeated freeze/thawing cycles of the reconstituted LAR II reagent decrease assay performance. Thus it is highly recommended to dispense this reagent into 100 μ L aliquots and store at -80°C . It is also recommended to thaw frozen aliquots of this reagent at room temperature.

receptors and transcription factors (1, 2) involved in global responses such as mitochondrial biogenesis(3-6) and tissue-specific responses such as adaptive thermogenesis(4), fatty acid oxidation(7, 8) , gluconeogenesis(9-11), and muscle fiber type switching(12). Alternative splicing of PPARGC1A gene produces a functional but predominantly cytosolic isoform of PGC-1 α (NT-PGC-1 α)(13) .NT-PGC-1 α retains the N-terminal transactivation and nuclear receptor interaction domains of PGC-1 α but lacks the C-terminal domains containing nuclear localization signals. NT-PGC-1 α enters the nucleus by passive diffusion but is actively translocated to the cytosol by CRM1-mediated nuclear export in basal conditions(14) . There are several precedents that transcriptional activity of transcription factors/coregulators are negatively regulated by sequestering from the nucleus. Their nuclear translocation is often controlled by multiple extracellular signals(15-19) . cAMP triggers nuclear accumulation of NT-PGC-1 α that is directly phosphorylated by cAMP-activated PKA at residues S194, S241, and T256 (13, 14).

To better understand direct correlation between PKA phosphorylation-dependent nuclear localization of NT-PGC-1 α and its enhanced coactivation capacity, we employed indirect immunofluorescence and luciferase reporter gene assay using HA-tagged wild type NT-PGC-1 α , phosphorylation-resistant NT-PGC-1 α -S194A/S241A/T256A, and phosphomimetic NT-PGC-1 α -S194D/S241D/T256D. Quantitative microscopic image analysis is critical to precisely determine signaling-dependent changes in subcellular localization of the protein of interest. In this chapter, we describe a fluorescence quantification method using Image J software to quantify the relative fluorescence intensity of the protein in the nucleus and cytoplasm in the single cell. Mean value of the relative nuclear/cytoplasmic fluorescence intensity ratios represents average subcellular localization of the protein in single cells. The frequency distribution histogram of nuclear/cytoplasmic intensity ratios provides the overview of signaling-dependent responses in the population of cells.

This chapter also describes transient cotransfection and dual-luciferase reporter assay, where the activities of firefly and *Renilla* luciferases are measured sequentially in a single sample. Firefly luciferase is used as an experimental reporter and *Renilla* luciferase is used as an internal control to measure transfection efficiency. The luciferase reporter gene assay, where the luciferase reporter gene is driven by a promoter of interest, is commonly used for studying the transcriptional activity of transcription factors on their responsive promoters. This assay is also applied to investigate how transcriptional coregulators modulate transcription factors. The Gal4-DBD, fused to the protein of interest or the protein domain of interest, is a useful tool to analyze transactivation property or identify functional domain of transcription factors/coregulators. The Gal4-DBD fusion protein is recruited to the nucleus due to strong nuclear localization signals in Gal4-DBD and binds to a Gal4-responsive element in a luciferase reporter pGK-1(20, 21).

2.Materials

2.1.CHO-K1 Cell Culture and Transfection

1. F-12K medium: F-12K supplemented with 1.26 g/L glucose and 2 mM glutamine.

2. F-12 + 10% FBS + 1% P/S: F-12K medium supplemented with 10% volume/ volume fetal bovine serum and 50 μ M penicillin and streptomycin.
3. 1 $\times$ Phosphate-buffered saline (PBS), pH, 7.4: 155.17 mM NaCl, 2.97 mM Na_2HPO_4 , 1.06 mM KH_2PO_4 .
4. Trypsin-EDTA: 0.5 g/L of trypsin and 0.2 g/L of EDTA·4Na in Hanks' Balanced Salt Solution without CaCl_2 , $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$.
5. Opti-MEM I Medium.
6. FuGENE® 6 Transfection Reagent. Store at 4°C.
7. Hemocytometer.
8. Inverted microscope.
9. 37°C water bath.

2.2. Immunofluorescence

1. Poly-L-lysine-coated coverslips (12 mm diameter) and microscope slides (25 $\times$ 75 $\times$ 1 mm).
2. 1 $\times$ PBS.
3. Paraformaldehyde: prepare fresh 4% (v/v) paraformaldehyde by diluting 16% paraformaldehyde solution with 1 $\times$ PBS.
4. Permeabilization solution: 0.5% (v/v) Triton X-100 in 1 $\times$ PBS.
5. 5 $\times$ Blocking solution: prepare 5% (v/v) normal goat serum (NGS) and 5% (v/v) bovine serum albumin, fraction V (BSA) in 1 $\times$ PBS and filter it through a 0.22 μ m filter unit, Durapore PVDF membrane (see Note 1).
6. HA-tag antibody.
7. Alexa Fluor 488-conjugated anti-rabbit immunoglobulin secondary antibody.
8. Vectashield mounting medium with DAPI (4, 6-diamino-2-phenylindole). Store at 4°C in the dark.
9. Nail polish.
10. Zeiss LSM510 confocal microscope.
11. Dibutyl cAMP: prepare 100 mM dibutyl cAMP in water.

2.3. Dual-Luciferase Reporter Assay

1. Dual-Luciferase® Kit.
2. BRL49653: prepare 10 mM BRL49653 in DMSO.
3. 9-cis-RA: prepare 10 mM 9-cis-RA in DMSO.
4. Dibutyl cAMP: prepare 100 mM dibutyl cAMP in water.
5. White polystyrene 96-well plate.

6. Luminometer.

3. Methods

3.1. CHO-K1 Cell Culture and Transfection

1. CHO-K1 cells are grown in F-12K + 10% FBS + 1% P/S medium in 10 cm² culture dishes at 37°C until 80% confluent.
2. Wash the cells with 1× PBS.
3. Add ~1 mL of pre-warmed trypsin-EDTA to the cells drop by drop and incubate the dish at 37°C incubator for 2–3 min.
4. Stop trypsin digestion by adding 5 mL of F-12K + 10% FBS + 1% P/S medium.
5. Count the number of cells per mL using hemocytometer and seed 3×10^4 cells per well in 2 mL in 12-well cell culture plate 1 day before transfection. Next day, confluence is ~40% (see Note 2).
6. Pipet 6 µL of FuGENE 6 Reagent directly into 94 µL of Opti-MEM I medium per well without contacting the walls of the plastic tube, mix gently, and incubate for 5 min at room temperature (see Note 3).
7. Add 1 µg of DNA into the tube, mix gently, and incubate for 30 min at room temperature.
8. Add the FuGENE 6 reagent: DNA complex to the cells in a drop-wise manner. Incubate the cells at 37°C overnight.
9. Replace the medium with fresh F-12K + 10% FBS + 1% P/S medium.

3.2. Immunofluorescence

All the fixation and immunolabeling procedures were performed in a humid chamber at room temperature.

1. Seed 3×10^4 CHO-K1 cells on top of poly-L-lysine-coated coverslip that sits in a well of 12-well culture plate 1 day before transfection.
2. Replace old F-12K + 10% FBS + 1% P/S medium with F-12K + 1% P/S medium 1 h before transfection (see Note 4).
3. Transfect CHO-K1 cells with wild type NT-PGC-1α-HA, NT-PGC-1α-S194A/S241A/T256A-HA, or NT-PGC-1α-S194D/S241D/T256D-HA using FuGENE 6 Transfection Reagent as described above.
4. 24 h after transfection, replace the medium with 1 mL of F-12K + 1% P/S medium containing vehicle or 500 µM dibutyryl cAMP (dbcAMP) and incubate for 1 h.
5. Wash the cells with 1× PBS three times.
6. Add 1 mL of 4% paraformaldehyde solution per well and fix the cells for 10 min, followed by three washes for 5 min each with 1× PBS.

7. Permeabilize with 0.5% Triton X-100 Permeabilization solution for 5 min.
8. Incubate the fixed cells with 5× Blocking solution for 30 min.
9. Incubate the fixed cells for 1 h with rabbit anti-HA antibody (1:4,000, Abcam) diluted in 1× Blocking solution and wash three times for 10 min each with 1× PBS.
10. Incubate the fixed cells for 1 h in the dark with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin secondary antibody (1:500) in 1× Blocking solution and wash three times for 10 min each with 1× PBS.
11. Put one drop of DAPI-containing Vectashield mounting medium on a microscope slide and carefully invert the coverslip into a drop of mounting medium. Gently press the coverslip to remove excess mounting medium. Seal the coverslip using nail polish when mounting medium is solidified. The sample can be viewed immediately after the nail polish is dry or be stored in the dark at 4°C.

3.3. Microscopy and Fluorescence Quantification

1. Acquire images using a Zeiss LSM 510 Meta confocal microscope (a Plan-Neofluar 40×/0.85 numerical aperture objective) coupled to a CCD camera. The same focal plane is used to obtain images of NT-PGC-1α (Alexa 488 channel) (Fig. 1a) and DAPI-stained nucleus (DAPI channel) in randomly selected positively transfected cells (150–200 cells).
2. To quantify the fluorescence signals in the nucleus and cytoplasm, the NIH Image J software is used. First, open the microscopic images obtained from the Alexa 488 and DAPI channels using Image J software and synchronize two windows. Manually outline the DAPI-stained nucleus using a free-hand selection tool and measure the selected nuclear area and integrated density of nuclear fluorescence signals. Similarly, outline the entire cell and measure the selected area of the cell and integrated density of total fluorescence signals.
3. Measure the fluorescence background from the cells that do not express NT-PGC-1α. Average these background signals and subtract them from the specific fluorescence signals for each NT-PGC-1α-expressing cell.
4. The area of the cytoplasm and cytoplasmic fluorescence signals are obtained by subtracting total nuclear area and fluorescence from total cellular area and fluorescence, respectively.
5. The relative nuclear/cytoplasmic (Nuc/Cyt) ratio of fluorescence intensity is determined by dividing the nuclear fluorescence intensity per unit area by the cytoplasmic fluorescence intensity per unit area (Fig. 1b). The nuclear/cytoplasmic intensity ratio of 1 represents even distribution of the protein between the nucleus and cytoplasm.
6. To determine overall responses of single cells to extracellular signals, create a frequency distribution histogram of the relative nuclear/cytoplasmic intensity ratios in the population of cells by plotting the number of cells vs. nuclear/cytoplasmic intensity ratios (Fig. 1c).

3.4. Dual-Luciferase Reporter Assay Using a (PPRE)₃-TK-luc Reporter

1. Seed 3×10^4 CHO-K1 cells per well in 12-well plate 1 day before transfection.
2. Transfect CHO-K1 cells with multiple plasmids (Table 1) using FuGENE 6 Transfection Reagent and incubate the cells in F-12K + 10% FBS + 1% P/S media.
3. 24 h after transfection, treat the cells with vehicle or $1 \mu\text{M}$ MBRL49653/ $1 \mu\text{M}$ 9-cis-RA for 6 h.
4. Wash the cells with $1 \times$ PBS.
5. Add 250 μL of $1 \times$ Passive Lysis Buffer (Promega Dual-Luciferase Reporter Assay Kit) to each well, place the 12-well culture plates on a rocking platform, and gently rock the plates for 15 min at room temperature.
6. Transfer the lysate to a microcentrifuge tube and centrifuge for 5 min. Transfer the supernatant to a new microcentrifuge tube.
7. Prepare Luciferase Assay Reagent II (LAR II) by dissolving the lyophilized Luciferase Assay Substrate in 10 mL of the Luciferase Assay Buffer II (see Note 5). LAR II should be prepared at ambient temperature just before use.
8. Prepare Stop & Glo Reagent (100 μL reagent per assay) by diluting $50 \times$ Stop & Glo Substrate to $1 \times$ with Stop & Glo Buffer. Stop & Glo Reagent should be prepared at ambient temperature just before use.
9. To carry out luciferase reporter assay, predispense 100 μL of LAR II into each well of a 96-well white plate. Transfer 20 μL of the cleared lysate and mix by pipetting two or three times. Place the plate in the luminometer and initiate firefly luciferase activity measurement.
10. Remove the plate from the luminometer and add 100 μL of Stop & Glo Reagent into each well. Place the plate in the luminometer and initiate *Renilla* luciferase activity measurement.
11. To normalize luciferase activity for transfection efficiency, divide the firefly luciferase activity measurements by *Renilla* luciferase activity measurements (Fig. 2a).
12. Perform at least two more independent luciferase reporter assays for each sample.

3.5. Dual-Luciferase Reporter Assay Using a pGK-1 luc Reporter

1. Seed 3×10^4 CHO-K1 cells per well in 12-well plate 1 day before transfection.
2. Transfect CHO-K1 cells with multiple plasmids (Table 2) using FuGENE 6 Transfection Reagent and incubate the cells in F-12K + 1% P/S medium for 24 h.
3. Treat the cells with vehicle or 500 μM dibutyryl cAMP for 1 h.
4. Follow steps 4–12 described in the Subheading 3.3 (Fig. 2b).

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Fig. 1.

PKA phosphorylation-dependent regulation of subcellular localization of NT-PGC-1 α . **(a)** Localization of NT-PGC-1 α -HA (WT), NT-PGC-1 α -S194A/S241A/T256A-HA, and NT-PGC-1 α -S194D/S241D/T256D-HA was analyzed in CHO-K1 cells that were treated with vehicle or 500 μ M dibutyryl cAMP (dbcAMP) for 1 h after serum-starvation. **(b)** Quantification of the relative nuclear to cytoplasmic fluorescence intensity ratios of NT-PGC-1 α wild type and mutants. The bar diagram of the relative ratios of nuclear to cytoplasmic fluorescence intensity is shown with the mean $\pm$ S.D. Significant difference by Student *t* test; ** $p < 0.01$. **(c)** Frequency distribution histogram of the relative nuclear/cytoplasmic intensity ratios in the population of cells. The number of cells was plotted over nuclear/cytoplasmic fluorescence intensity ratios. A significant shift to higher ratios is shown in the population of cells by dibutyryl cAMP treatment and PKA phosphorylation.

Fig. 2. Correlation of increased coactivation capacity of NT-PGC-1 α with its nuclear concentration. **(a)** Transcriptional coactivation assay. pcDNA3.1, NT-PGC-1 α (WT), NT-PGC-1 α -S194A/S241A/T256A (Ala), or NT-PGC-1 α -S194D/S241D/T256D (Asp) were cotransfected into CHO-K1 cells with a PPRE luciferase reporter [(PPRE)₃-TK-luc], RXR α , PPAR γ , and a *Renilla* luciferase reporter. Twenty-four hour after transfection, cells were treated with vehicle or 1 μ M BRL49653/1 μ M 9-cis-RA for 6 h. The relative luciferase activity units were determined by dividing firefly luciferase activity measurements by *Renilla* luciferase activity measurements. Data represent mean $\pm$ S.D. of at least three independent experiments. Significant difference is determined by student *t* test; **p* < 0.05. Increased nuclear localization of NT-PGC-1 α -S194D/S241D/T256D enhances its coactivation capacity. **(b)** Transcriptional activity assay. Gal4-DBD, Gal4-DBD-NT-PGC-1 α (WT), Gal4-DBD-NT-PGC-1 α -S194A/S241A/T256A (Ala), and Gal4-DBD-NT-PGC-1 α -S194D/S241D/T256D (Asp) were cotransfected into CHO-K1 cells with a pGK-1 luciferase reporter containing Gal4 DNA binding sites and a *Renilla* luciferase reporter. 24 h after serum-starved transfection, cells were treated with vehicle or 500 μ M dibutyryl cAMP (dbcAMP) for 1 h. Luciferase activity was determined as described above. Data represent mean $\pm$ S.D. of at least five independent experiments. All the Gal4-DBD fusion proteins are targeted to the nucleus. PKA phosphorylation does not increase the transcriptional activity itself of NT-PGC-1 α .

Table 1
Cotransfection of CHO-K1 cells for transcriptional coactivation assay using a (PPRE)₃-TK-luc reporter

Samples 1	1	2	3	4	5	6	7	8
(PPRE) ₃ -TK-luc	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
pRL-SV40	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
pSV sport-PPAR γ	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
pSV sport-RXR α	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
pcDNA3.1	0.3	0.3						
pcDNA3.1-NT-PGC-1 α			0.3	0.3				
pcDNA3.1-NT-PGC-1 α -S194A/S241A/T256A					0.3	0.3		
pcDNA3.1-NT-PGC-1 α -S 194D/S241D/T256D							0.3	0.3
BRL49653 + 9-cis-RA	-	+	-	+	-	+	-	+
Total μ g	1	1	1	1	1	1	1	1

Table 2
Cotransfection of CHO-K1 cells for transcriptional activity assay using a pGK-luc reporter

Samples	2	1	2	3	4	5	6	7	8
pGK-luc		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
pRL-SV40		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
pCMV-DBD		0.1	0.1						
pCMV-DBD-NT-PGC-1 α				0.1	0.1				
pCMV-DBD-NT-PGC-1 α -S194A/S241A/T256A						0.1	0.1		
pCMV-DBD-NT-PGC-1 α -S194D/S241D/T256D								0.1	0.1
Dibutyryl cAMP		–	+	–	+	–	+	–	+
Total μ g		0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65